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(57) Abstract

Avidin and streptavidin have been found to be larvicidal against a number of common insect pests of agricultural crops and stored grains. In a preferred embodiment, plant resistance to these insects is produced by inserting into the cells of a plant a gene whose expression causes production of one or more of these glycoproteins in larvicidal amounts.

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AVIDIN & HOMOLOGUES AS LARVICIDES AGAINST INSECT PESTS

Technical Field

This invention relates to materials and methods for killing insect larvae which are harmful to plants, and materials and methods for imparting insect resistance to plants, material harvested from the plants, and products derived from the harvested material.

Background of the Invention

Numerous insects are serious pests of common agricultural crops. One method of controlling insects has been to apply insecticidal organic or semiorganic chemicals to crops. This method has numerous, art-recognized problems. A more recent method of control of insect pests has been the use of biological control organisms which are typically natural predators of the troublesome insects. These include other insects, fungi (milky-spore) and bacteria (*Bacillus thuringiensis* cv., commonly referred to as "Bt"). However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express insecticidal toxins derived from biological control organisms such as Bt. This technology has given rise to additional concerns about eventual insect resistance to well-known, naturally occurring insect toxins, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing need exists to identify naturally occurring insecticidal toxins which can be formed by plant cells directly by translation of a single structural gene.

Brief Description of the Drawings

Figure 1 illustrates the gene map of plasmid pPHI414 which is useful as an expression cassette for protein structural genes.

Figure 2 illustrates the gene map of plasmid pPHI412 which is also useful as an expression cassette for protein structural genes.

Disclosure of the Invention

It has now been determined that the glycoprotein avidin and the protein streptavidin have potent larvicidal activity when administered enterally to the larvae of insects such as European corn borer, corn rootworm, red flour beetle, alfalfa weevil, tobacco budworm, beet armyworm, bollworm, sunflower moth, confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth. Thus, this invention provides a method for killing susceptible insect larvae, including larvae selected from European corn borer, corn rootworm, red flour beetle, alfalfa weevil, tobacco budworm, beet armyworm, bollworm, sunflower moth, confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth and comprising administering enterally to the larvae a larvicidal amount of a protein or glycoprotein selected from avidin and streptavidin, and proteins and glycoproteins at least 90% homologous thereto by amino acid sequence.

Avidin was originally a factor isolated from raw egg white as described by Eakin et al., J. Biol. Chem., 136, 801 (1940); Pennington et al., J. Am. Chem. Soc., 64, 469 (1942); Fraenkel-Conrat et al., Arch. Biochem. Biophys., 39, 80, 97 (1952). Later, improved methods of purification were disclosed by Green et al., Biochem. J., 118, 67, 71 (1970). The glycoprotein contains four essentially identical subunits having a combined molecular weight of about 66,000 daltons. Each subunit is a single polypeptide chain containing 128 amino acid residues with alanine at the N-terminus, glutamic acid at the C-terminus, and a

carbohydrate moiety attached at the asparaginyl residue at position 17 through post-translational processing. The complete amino acid sequence has been determined and has been published. See, e.g., DeLange, H., J. Biol. Chem., 246, 698 (1971). The bacterial version, streptavidin, is not glycosylated. The larvicidal compound can be effectively applied to plants, harvested materials, or products consumed by the larvae by spray, dust or other formulation common to the insecticidal arts. By "harvested plant material" herein is meant any material harvested from an agricultural or horticultural crop, including without limitation grain, fruit, leaves, fibers, seeds, or other plant parts. Products derived or obtained from such harvested material include flour, meal, and flakes derived from grain and products in which such materials are admixed, such as, for example, cake, pancake and biscuit mixes. Alternatively, the larvicidal protein or glycoprotein can be incorporated into the tissues of a susceptible plant so that in the course of infesting the plant, its harvested material, or a product derived from harvested plant material, the larvae consume larvicidal amounts of the protein or glycoprotein. One method of doing this is to incorporate the compound in a non-phytotoxic vehicle which is adapted for systemic administration to the susceptible plants. This method is commonly employed with insecticidal materials which are designed to attack chewing insects and is well within the purview of one of ordinary skill in the art of insecticide and larvicide formulation. However, since the genes which code for these peptides can be isolated and cloned, or can be synthesized directly using a DNA sequence obtained by working backwards from the known amino acid sequence and preferably using plant-preferred codons. The resulting sequence can be inserted into an appropriate expression cassette, and introduced into cells of a susceptible plant species, so that an especially preferred mbodiment of this method involves inserting into the genome of the plant a DNA sequence coding for one or more ins cticidal plant proteins or glycoproteins selected

from avidin and streptavidin and proteins and glycoproteins having at least 90% homology thereto by amino acid sequence, in proper reading frame relative to transcription initiator and promoter sequences active in the plant. Transcription and translation of the DNA sequence under control of the plant-active regulatory sequences causes expression of the larvicidal gene product at levels which provide an insecticidal amount of the compound in the tissues of the plant which are normally infested by the larvae. Alternatively, a dietary bait containing one or more of the selected compounds can be employed, with, optionally, an added pheromonal or other larval attractant material.

The plant is preferably a plant susceptible, or whose harvested material or products are susceptible to infestation and damage by the larvae of one or more insect larvae selected from European corn borer, corn rootworm and alfalfa weevil, sunflower moth, bollworm, tobacco budworm, and beet armyworm or whose harvested material is subject to attack by red flour beetle, confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth. These include corn (Zea mays), wheat (Triticum aestivum) and sorghum (Sorghum bicolor). However, this is not to be construed as limiting, inasmuch as these species are among the most difficult commercial crops to reliably transform and regenerate, and these insects (under other common names) also infest certain other crops. Thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, if they are found to be susceptible to the plant pests listed hereinabove, including, without limitation, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manicot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hemerocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browallia,

Glycine, Lolium, Triticum, and Datura.

Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean, fiber crops, such as cotton, fruit crops, such as melons, and vegetable crops, including onion, pepper, tomato, cucumber, squash, carrot, crucifer (cabbage, broccoli, cauliflower), eggplant, spinach, potato and lettuce.

The DNA sequence which when expressed imparts insecticidal activity is a structural gene which codes for avidin, streptavidin, or a protein or glycoprotein having at least 90% homology to avidin or streptavidin. It has been found that these compounds have sufficient insecticidal (larvicidal) activity to be operative in a plant cell expression system. That is, while certain other proteins have some larvicidal activity at high concentrations in pure form, plant cell expression at such high concentrations is either not possible in a living plant cell system, or is not feasible if the commercially useful characteristics of the plant are to be preserved in terms of production of oils, starches, fibers, or other materials. A tissue specific promoter can be used in any instance where it may be desirable to localize production of the compound to an infested tissue or to a tissue which is efficient in production of the protein or glycoprotein.

In carrying out this invention, it will be appreciated that numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression

cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the DNA sequence of the present invention can be inserted is the pPHI414 plasmid developed by Beach et al. of Pioneer Hi-Bred International, Inc., Johnston, IA. Highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve as selectable markers to identify and separate transformed cells. Preferred selection agents include kanamycin, chlorosulfuron, phosphonothricin, hygromycin and methotrexate, and preferred markers are genes conferring resistance to these compounds. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant".

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule that is not a part of the chromosomes of the cell.

As mentioned above, genomic, synthetic and cDNA encoding the gene of interest may be used in this invention. The vector of interest may also be constructed partially from a cDNA clone, partially from a synthetic sequence and partially from a genomic clone. When the gene sequence of interest is in hand, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell.

According to this invention, the genetic construct will contain (a) a first genetic sequence coding for the protein or glycoprotein of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will be selected from the group comprising of promoters and terminators. The regulatory sequences may be from autologous or heterologous sources.

Promoters that may be used in the genetic sequence include nos, ocs, phaseolin, CaMV, FMV and other promoters isolated from plants or plant pests.

An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-biphosphate carboxylase from soybean (Berry-Lowe et al, <u>J. Molecular and App. Gen.</u>, <u>1</u>:483-498 (1982)), and the promoter of the cholorophyll a-b binding protein. These two promoters are known to be light-induced, in eukaryotic plant cells (see, for example, <u>Genetic Engineering of Plants, An Agricultural Perspective</u>, A. Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi et al., <u>J. Biol. Chem.</u>, <u>258</u>:1399 (1983), and P. Dunsmuir, et al., <u>J. Molecular and App. Gen.</u>, <u>2</u>:285 (1983)).

The expression cassette comprising the structural gene for the compound of interest operably linked to the desired control sequences can be ligated into a suitable cloning vector. In general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

Typically, an intermediate host cell will be used in

the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells. Host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as E. coli, S. typhimurium, and S. marcescens. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention.

The isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, microparticle bombardment, and microinjection, into cells from monocotyledonous or dicotyledonous plants, in cell or tissue culture, to provide transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of the plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, wheat and rice, and the dicotyledonous species will be selected from soybean, sunflower, cotton, rapeseed (either edible or industrial), alfalfa, tobacco, and Solanaceae such as potato and tomato. Using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the desired gene for the selected protein. Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette of this invention.

This invention also provides methods of imparting resistance to insects selected from European corn borer, corn rootworm, red flour beetle and alfalfa weevil, tobacco budworm, beet armyworm, bollworm, sunflower moth, confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth to plants of a susceptible taxon, comprising the steps of:

a) culturing cells or tissues from at least one plant

from the taxon,

b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a structural gene coding for a protein or glycoprotein selected from avidin, streptavidin and proteins and glycoproteins having at least 90% homology thereto by amino acid sequence, or a combination of such proteins, operably linked to plant regulatory sequences which cause the expression of the protein structural gene in the cells, and

c) regenerating insect-resistant whole plants from the cell or tissue culture. Once whole plants have been obtained, they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

Alternatively, once a single transformed plant has been obtained by the foregoing recombinant DNA method, conventional plant breeding methods can be used to transfer the protein structural gene and associated regulatory sequences via crossing and backcrossing. Such intermediate methods will comprise the further steps of

a) sexually crossing the insect-resistant plant with a plant from the insect-susceptible taxon;

b) recovering reproductive material from the progeny of the cross; and

c) growing insect-resistant plants from the reproductive material. Where desirable or necessary, the agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

a) backcrossing the insect-resistant progeny with insect-susceptible plants from the susceptible taxon; and

b) selecting for expression of insect resistance (or an associated marker gene) among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting insect resistance.

By the term "taxon" herein is meant a unit of botanical

classification of genus or lower. It thus includes genus, species, cultivars, varieties, variants, and other minor taxonomic groups which lack a consistent nomenclature.

It will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into Agrobacterium tumefaciens, which can then be used to transfer the vector into susceptible plant cells, primarily from dicotyledonous species. Thus, this invention provides a method for imparting insect resistance in Agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with Agrobacterium tumefaciens, a plasmid of which has been modified to include a plant expression cassette of this invention.

Finally, it has also been determined that these compounds can exert their larvicidal activity against insect pests of harvested material, including stored grain, such as the red flour beetle (Tribolium castanaeum). Thus, these insects can also be targets for the compositions and methods of this invention. In view of this, the invention also provides a methods and compositions for killing larvae of red flour beetle and confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth and other susceptible insect pests of harvested materials and products obtained from harvested materials, comprising applying to the grain or causing to be expressed in the grain a protein or glycoprotein selected from avidin, streptavidin, or a protein or glycoprotein having at least 90% homology thereto by amino acid sequence.

The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

In the following examples the wide variety of insects screened has resulted in several different bioassays being used to determine the effect of avidin and streptavidin on larval growth and survivorship. However, all of the

bioassays allow the protein to be enterally administered to the insect. In vitro bioassays for the European corn borer (Ostrinia nubalis), southern corn rootworm (Diabrotica undecimpunctata howardi), sunflower moth (Homoeosoma electellum), black cutworm (Agrotis ipsilon), tobacco budworm (Helicoverpa virescens), beet armyworm (Spodoptera exigua), and bollworm (Helicoverpa zea) were done by incorporating the test protein into the artificial diet. This is referred to herein as an "Incorporated Bioassay". This was accomplished by making up a standard artificial diet at 90% of the original water and adding a solution of the test protein to this mixture. Concentrations of the protein in this diet are recorded as mg or μg of protein per ml of diet. A modification of this test involves preparing the original diet, then preparing a 1% solution of the test protein that is applied (75 μl) to the diet surface. This is referred to herein as a "Topical bioassay," but it should be understood that the test compound is applied topically to the insect's diet, not to the insect itself. Weight and mortality are recorded after seven days. Specific assays and variations are described in the individual examples.

Example 1

EUROPEAN CORN BORER

Avidin and streptavidin were very effective against European corn borer with high mortality occurring during a 7-day topical bioassay. The results are shown in Table 1.

Table 1.

Effect of avidin and streptavidin on European corn borer neonate larvae in topical bioassays

Treatment	Weight (mg)	Mortality
Control	9.4	0/16
Avidin 1%	--	16/16
Streptavidin	2.5	15/16

Avidin and streptavidin were also effective when incorporated into the artificial diet. Larvae were reared

on diets containing 0.125, 0.25, 0.5 and 1 mg/ml of avidin or streptavidin. Results are shown in Table 2. All the concentrations tested displayed mortalities greater than the control and survivors had greatly reduced larval weights.

Table 2

Effect of avidin and streptavidin on neonate European corn borer larvae in incorporated bioassays

Treatment (mg/ml)	Weight (mg)	Mortality
Control	5.8	1/16
Avidin 1	1.4	8/16
Avidin 0.5	1.3	10/16
Avidin 0.25	2.0	12/16
Avidin 0.125	1.5	6/16
Strept. 1	--	16/16
Strept 0.5	2.3	12/16
Strept 0.25	2.0	4/16
Strept 0.125	1.6	12/16

ECB larvae survivors reared on an avidin-containing diet can be rescued by the addition of biotin to the diet.

Example 2

SOUTHERN CORN ROOTWORM

Avidin and streptavidin have not had as dramatic effect an on southern corn rootworm larvae. Avidin shows an increase in mortality at 10 mg/ml, but no effect is seen at 1 mg/ml Results are shown in Table 3.

Table 3

Effect of avidin on neonate SCR larvae in incorporated bioassays

Treatment (mg/ml)	Weight (mg)	Mortality
Control	2.4	0/8
Avidin 10	1.7	4/8
Avidin 5	1.6	3/8
Avidin 1	2.0	1/8

Examples 3-5

BOLLWORM, TOBACCO BUDWORM, BEET ARMYWORM, SUNFLOWER MOTH, BLACK CUTWORM

Avidin was effective against bollworm, tobacco budworm and beet army worm at 1 mg/ml. Both high mortality and reduced growth rate were observed. Results are tabulated in Table 4.

Table 4

Percent mortality and larval weight reduction scores after 7 days for bollworms (BW), tobacco budworms (TBW), black cutworms (BCW), sunflower moths (SM) and beet armyworms (BAW) reared on avidin (1 mg/ml) diet when compared to the control

	%mortality (Wt. Reduction)				
Insect	BW	TBW	BCW	SM	BAW
	97 (7)	75 (7)	95(8)	100(-)	81 (9)

Wt. Reduction: The -fold reduction in larvae weight as compared to the control diet.

Example 6

POTATO LEAFHOPPER

Avidin had no effect on female adult potato leafhoppers (data not shown).

Example 7

ALFALFA WEEVIL

In vitro bioassays for the alfalfa weevil (Hypera postica) used alfalfa leaflets that were painted with a 2% solution of the test protein. In each test, two leaflets and one neonate larvae were placed in a bioassay well. Weight, % consumption and mortality were recorded after seven days.

Alfalfa weevil was affected by avidin. Larvae reared on leaflets painted with avidin had a higher mortality, and survivors weighed slightly less and consumed less leaf material. Results, representing the means of 4 tests, are presented in Table 5.

Table 5

Percent leaf consumption, weight and percent mortality for alfalfa weevil larvae fed on fresh leaf tissue with a 2% topical application of avidin

Treatment	%consumption	Mean wt (mg)	% Mortality
Control	80	3.1	11
Avidin	31	1.8	83

Examples 8-13

RED FLOUR BEETLE, CONFUSED FLOUR BEETLE, LESSER GRAIN BORER, SAWTOOTHED GRAIN BEETLE, RICE WEEVIL, INDIANMEAL MOTH

In vitro bioassay for the red flour beetle (Tribolium castaneum), confused flour beetle (Tribolium confusum), lesser grain borer (Rhyzopertha dominica), sawtoothed grain beetle (Oryzaephilus surinamensis), rice weevil (Sitophilus oryzae) and Indianmeal moth (Plodia interpunctella) use diets made of wheat germ or cracked wheat. The protein is incorporated into the diet and frozen overnight. The frozen sample is lyophilized for 24 hours, pulverized into a powder, and equilibrated in a 75% relative humidity chamber for 1 week. Larvae are then used to infest the diet. Weight, mortality, insect sounds and adult emergence can be measured at different times.

Example 8

Indianmeal moth

Avidin reduced weight gain and disrupted pupation of red flour beetle larvae. Results can be seen in Table 6. Only 1 larvae reached pupation at the higher levels of avidin.

Table 6

Eff ct of avidin on growth and pupation of the r d flour beetle reared on artificial diet.

Treatment (%)	Weight (mg, 18 days)	%pupation
Control	2.5	10/10
Avidin 0.001	2.6	9/10
Avidin 0.01	1.3	1/10
Avidin 0.1	0.7	0/10

Addition of biotin to the diet negated the effects avidin had on the red flour beetle.

Examples 9-10

Avidin had a similar effect on the growth of the confused flour beetle and sawtoothed grain beetle. After 15 days, confused flour beetle larvae reared on control and Avidin 0.1% + biotin diets weighed approximately 2.9 mg, while larvae reared on 0.1% avidin only weighed 1.0 mg. Sawtoothed grain beetle larvae reared on control diet weighed approximately 0.6 mg, while larvae reared on 0.1% avidin only weight 0.1 mg.

Examples 11-13

Avidin was very effective in reducing adult emergence and number of feeding sounds produced by the rice weevil, lesser grain borer and Indianmeal moth. Results are shown in Table 7.

Table 7

Effect of avidin on the development of the rice weevil (RW), lesser grainborer (LGB) and Indianmeal moth (IM).

	RW		LGB		IM	
Treatment (%)	sounds	adults	sounds	adults	sounds	adults
Control (0%)	9,500	57	19,500	53	50,000	N.A.
Avid. 0.001	13,000	48	3,500	17	N.A.	N.A.
Avid. 0.01	170	0	30	0	N.A.	N.A.
Avid. 0.1	N.A.	N.A.	N.A.	N.A.	5,000	N.A.

To date, avidin has been effective against all insect pests of stored grain and products obtained from stored grain. Avidin has reduced growth rate, increased time required for development, increased mortality and decreased

the amount of insect feeding as determined by crunching sounds produced by feeding insects, recorded using ultrasensitive sound equipment.

Industrial Applicability

I. Isolation of the protein gene and insertion into bacteria

In order to isolate the coding sequence for the protein, it is necessary to have nucleotide sequence data which establishes an open reading frame (i.e., the correct triplet code for translation which should have only one "stop" signal at the very end of the gene.) It is also necessary to have an indication of where to look for the protease cleavage junction between the protein and the replicase which precedes it in the sequence. This can be determined from the peptide sequence of the N-terminal portion of the protein or by comparing the protein sequence with that of other homologous proteins. This can generally be accomplished and the necessary information obtained without sequencing the entire gene. Once the sequence at both ends of the gene has been determined, the remainder of the gene can be cloned using restriction enzymes that flank the protein coding region or, more preferably, by cloning the precise protein coding region by oligonucleotide-directed amplification of DNA (polymerase chain reaction or PCR).

Once the gene has been isolated, it can be cloned into a bacterial expression vector with linkers added to create all three reading frames (using 8mer, 10mer, and 12mers each of which contain an ATG translational start site). The resulting vectors, containing the fragments of interest, can be inserted into, for example, BRL's Maximum Efficiency DH5 F' IQ transformation competent _E. coli_ cells. All three transformations, one for each linker, are then screened via minipreps for the presence and orientation of insert. Appropriate clones are then chosen to test for expression of the protein gene.

Clones containing the properly oriented inserts are

grown in culture medium conducive to the induction of the gene (LB medium with added IPTG). The cells are lysed and bacterial proteins are subjected to electrophoresis in SDS polyacrylamide gels and then transferred to nitrocellulose. The resulting protein blots are easily screened for presence of protein using rabbit polyclonal and mouse monoclonal anti-protein antibody.

Having determined the proper reading frame, it is then necessary to remove the gene from the bacterial expression vector. The linker at the start of the gene region supplies the necessary start codon.

II. Expression of the Protein Gene in Plants

A plant expression cassette, employing the regulatory sequences developed by Beach, et al., and containing the protein gene, is constructed. The restriction map of the preferred plasmid, designated pPHI414, is illustrated in Figure 1. This plasmid contains an enhanced 35S promoter spanning nucleotides - 421 to +2 of Cauliflower Mosaic Virus with the region from - 421 to - 90 duplicated in tandem, a 79 bp HindIII Sall fragment from pJII101 spanning the 5' leader sequence of Tobacco Mosaic Virus, a 579 bp fragment spanning the first intron from maize AdH1-S, and a 281 bp fragment spanning the polyadenylation site from the nopaline synthase gene in pTiT37.

Another construct which can be used as an expression cassette is the pPHI412 plasmid shown in Figure 2. It differs from pPHI414 in that it lacks the AdH intron segment. However, like pPHI414, it is constructed to have numerous restriction sites between the O' segment and the NOS segment, which sites can be conveniently used for splicing any desired protein structural gene into position.

This vector can be cotransformed with a similar plasmid containing a selectable marker for antibiotic resistance into Black Mexican Sweet corn protoplasts by electroporation. These protoplasts can then be induced to regenerate cell walls and develop into callus by conventional techniques. Likewise, this callus can then be

subjected to antibiotic selection to select for transformed colonies, and these colonies can be tested for expression of protein with antisera for the appropriate protein using known methods. The efficiency of protection can be measured by infesting callus (or suspension cultures derived from callus) with the target insect and measuring survival percentages.

The protein gene can be introduced into embryogenic maize callus by methods similar to those used for Black Mexican Sweet. Embryogenic callus can be regenerated to whole fertile plants. The insect resistance imparted by the endogenous production of the protein is a simply inherited, dominant trait and can, if desired, be introduced into other plant varieties of the species by simple crossing or backcrossing.

Using the foregoing techniques, avidin has been expressed in maize suspension cells as determined by transient assays.

WHAT IS CLAIMED IS:

1. A method for killing insect larvae selected from European corn borer, corn rootworm, red flour beetle and alfalfa weevil, tobacco budworm, beet armyworm, bollworm, sunflower moth, confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth, comprising administering enterally to the larvae a larvicidal amount of avidin, streptavidin, a protein or glycoprotein having at least 90% homology thereto, or a combination thereof.

2. A method according to Claim 1 wherein the protein, glycoprotein or combination is administered enterally by incorporating the protein glycoprotein or combination in the diet of the larvae.

3. A method according to Claim 2 wherein the diet of the larvae comprises the tissues of a living plant.

4. A method according Claim 3 wherein the protein, glycoprotein or combination comprises a protein which is not native to the plant.

5. A method according to Claim 1 for protecting a plant, harvested material from the plant, and products derived from the harvested material against infestation by insect larvae selected from European corn borer, corn rootworm, red flour beetle and alfalfa weevil, tobacco budworm, beet armyworm, bollworm, sunflower moth, confused flour beetle, black cutworm, lesser grain borer, sawtoothed grain beetle, rice weevil and Indian meal moth and comprising inserting into the genome of the plant at least one sequence coding for avidin, streptavidin, a protein or glycoprotein having at least 90% homology thereto, or a combination of such proteins or glycoproteins, in proper reading frame relative to transcription initiator and promoter sequences active in the plant to cause expression of the sequence or sequences at levels which provide a larvicidal amount of the gene product in the tissues of the plant or harvested material of the plant which are normally infested by the larvae.

6. A method according to Claim 5 wherein the plant is a monocotyledonous species selected from corn, wheat, rice and sorghum.

7. A method according to Claim 5 wherein the plant is a dicotyledonous species selected from soybean, sunflower, rapeseed, alfalfa, cotton and tomato.

8. A method according to Claim 5 further comprising the steps of:

a) culturing cells or tissues from the plant,

b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a sequence coding for the protein or combination of proteins, and

c) regenerating resistant whole plants from the cell or tissue culture.

9. A method according to Claim 8 which comprises the further step of sexually or clonally reproducing the whole plant in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

10. A method according to Claim 8 in which the expression cassette is introduced into the cells by electroporation.

11. A method according to Claim 8 in which the expression cassette is introduced into the cells by microparticle bombardment.

12. A method according to Claim 8 in which the expression cassette is introduced into the cells by microinjection.

13. A method according to Claim 8 for providing resistance to insects in <u>Agrobacterium tumefaciens</u>-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with <u>Agrobacterium tumefaciens</u>, a plasmid of which has been modified to include the expression cassette.

14. A method of imparting resistance to insects selected from European corn borer, corn rootworm, and alfalfa weevil, sunflower moth, bollworm, tobacco budworm,

and beet armyworm to plants of a taxon susceptible to those insects, and thereby to harvested material from the plants and products obtained from the harvested material, comprising the steps of:

a) selecting a fertile, insect resistant plant prepared by the method of Claim 8 from a sexually compatible species;

b) sexually crossing the insect resistant plant with a plant from the insect susceptible taxon;

c) recovering reproductive material from the progeny of the cross; and

d) growing resistant plants from the reproductive material.

15. A method according to Claim 14 for imparting insect resistance in a taxon consisting of substantially homozygous plants, and thereby to harvested material from the plants and products obtained from the harvested material, which comprises the further steps of repetitively:

a) backcrossing the insect resistant progeny with substantially homozygous, insect susceptible plants from the taxon; and

b) selecting for expression of both insect resistance and the other characteristics of the susceptible taxon among the progeny of the backcross,until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with insect resistance.

16. An isolated DNA sequence which codes substantially solely for avidin, streptavidin, a glycoprotein having at least 90% homology thereto, or a combination of such glycoproteins.

17. An expression cassette comprising a DNA sequence according to Claim 16 operably linked to plant regulatory sequences which cause the expression of the DNA clone in plant cells.

18. An expression cassette comprising a DNA sequence according to Claim 16 operably linked to bacterial expression regulatory sequences which cause the expression of the DNA clone in bacterial cells.

19. Bacterial cells containing as a foreign plasmid at least one copy of an expression cassette according to Claim 18.

20. Transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of an expression cassette according to Claim 17.

21. Transformed cells according to Claim 20, further characterized in being cells of a monocotyledonous species.

22. Transformed cells according to Claim 21, further characterized in being maize, sorghum, wheat or rice cells.

23. Transformed cells according to Claim 20, further characterized in being cells of a dicotyledonous species.

24. Transformed cells according to Claim 23, further characterized in being soybean, alfalfa, sunflower, rapeseed, cotton or tomato cells.

25. A maize cell- or tissue-culture comprising cells according to claim 23.

26. A transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette according to Claim 17.

27. A larvicidal composition, comprising a larvicidal amount of avidin, streptavidin, a protein or glycoprotein having at least 90% homology thereto, or a combination of such proteins or glycoproteins in a non-phytotoxic vehicle.

28. A composition according to Claim 27 wherein the vehicle is adapted for systemic administration to a susceptible plant.

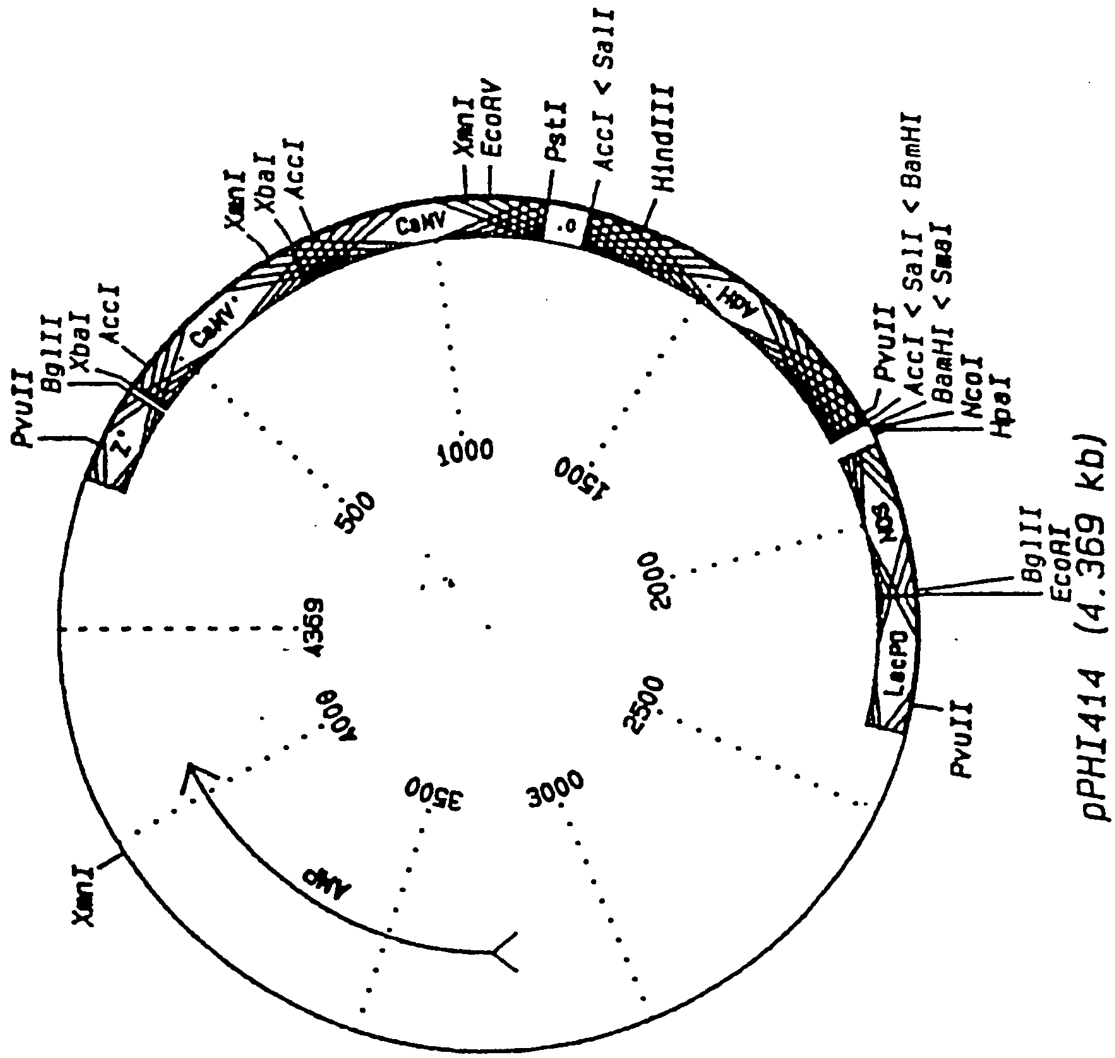
29. A composition according to Claim 27 wherein the vehicle further comprises a larval dietary bait for susceptible insects.

30. A composition according to Claim 29 wherein the bait further comprises a pheromonal larval attractant for susceptible insects.

31. A method of killing or controlling insect pests of harvested plant material, comprising applying to the harvested material a composition comprising avidin, streptavidin, a protein or glycoprotein having at least 90% homology thereto, or a combination thereof.

32. A method of killing or controlling insect pests of harvested plant material, comprising incorporating into the harvested materials avidin, streptavidin, a protein or glycoprotein having at least 90% homology thereto, or a combination thereof.

33. A method according to Claim 32 wherein the insect pest is the red flour beetle.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EcoRV
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AccI < SalI < BamHI
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XmnI
PvuII
BglII
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AccI
CaMV
CaMV
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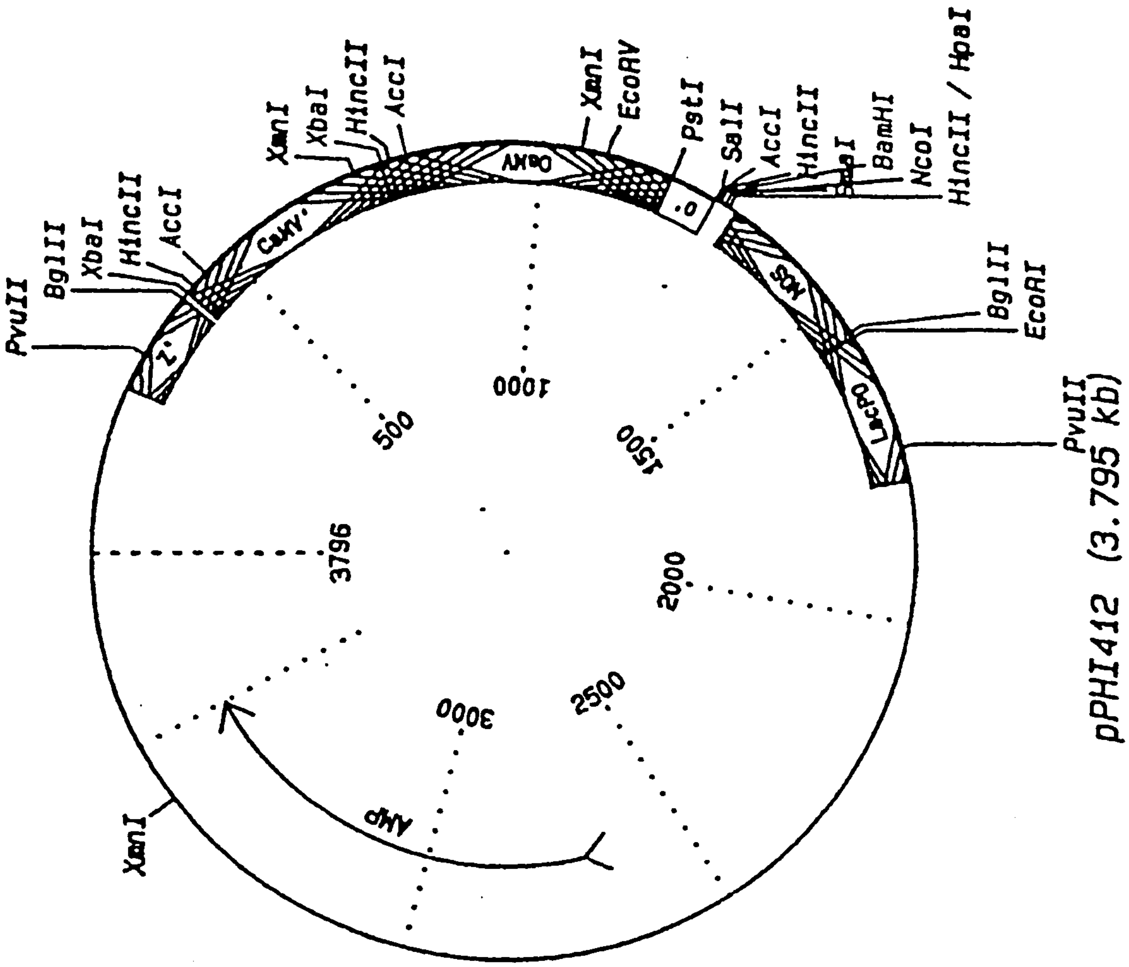
4000
4369

Fig. 1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BglII
XbaI
HincII
AccI
XmnI
XbaI
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AccI
XmnI
EcoRV
PstI
SalI
AccI
HincII
BamHI
NcoI
HincII / HpaI
BglII
EcoRI
PvuII
Z'
CaMV
NOS
LacPO
500
1000
1500
2000
2500
3000
3796
AMP
XmnI
pPHI412 (3.795 kb)

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/06487

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A01N63/02 C12N15/12 C12N15/31 C12N15/74 C12N15/82
C12N1/21 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INSECT BIOCHEMISTRY vol. 21, no. 5 , 1991 , OXFORD,GB pages 535 - 539 B.G. BRUINS ET AL. 'the harmful effect of light on drosophila is diet-dependent' see page 537, column 1, last paragraph ---	1-4, 27-33
X	WO,A,86 02077 (H.M. MEADE ET AL.) 10 April 1986 see claims 1-10 ---	16-20
X	WO,A,87 05026 (THE TRUSTEES OF COLUMBIA UNIVERCITY IN THE CITY OF NEW YORK) 27 August 1987 see claims 1-13 --- -/--	16,18,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
27 Oct ber 1993	0 3. 11. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	DECORTE, D

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 03422 (BRITISH BIO-TECHNOLOGY) 20 April 1989 see claims -----	16,18,19

3

INTERNATIONAL SEARCH REPORT

...formation on patent family members

International application No.
PCT/US 93/06487

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		JP-T-	62500700	26-03-87
		US-A-	5168049	01-12-92
WO-A-8705026	27-08-87	US-A-	4839293	13-06-89
		AU-A-	7165287	09-09-87
		EP-A-	0258411	09-03-88
		JP-T-	63502560	29-09-88
WO-A-8903422	20-04-89	EP-A-	0390785	10-10-90
		JP-T-	3501923	09-05-91